Supporting Information

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SI Materials and Methods

Constructs and Reagents. A combined luciferase reporter construct containing both miR-184 and miR-205 consensus target sequences (184/205_PER), which serves as a positive control, was made in pMIR-Report (Ambion). Top (CTAGTAATATTACCCTTATCAGTTCTCCGTCCCAGACTCCGGTGGAATGAAGGA) and bottom (AGCTTCCTTCATTCCACCGGAGTCTGGGACGGAGAACTGATAAGGGTAATATTA) strand oligonucleotides specifying the 184 target sequence directly followed by the 205 target sequence and containing SpeI and HinDIII linkers at the 5′ and 3′ ends, respectively, were annealed and ligated to the SpeI and HinDIII sites of pMIR-Report. The 3′ UTR of the human SHIP2 mRNA was generated by RT-PCR and TA cloned into pCR2.1 (Invitrogen). The SHIP2 3′UTR sequence was verified and was subsequently cloned in between the SpeI and HinDIII sites of pMIR-Report.

Antagomirs directed against miR-184, miR-205, and miR-124 were synthesized by Dharmacon according to the following structural specifications: antagomir-184, 5′-AsCsCsCUUAUCAGUUCUCCGUsCsCsA-Chol-3′; antagomir-205, 5′-CsAsGsACUCCGGUGGAAUGAAsGsGsA-Chol-3′; antagomir-124, 5′-GsGsCsAUUCACCGCGUGCsCsUsU-Chol-3′. Uppercase letters represent 2′-OMe-modified nucleotides, "s" represents a phosphorothioate linkage, and Chol represents cholesterol.

Immunohistochemistry and Light microscopy. HeLa, HEK, and HCEK cultures grown on glass coverslips were fixed in 4% paraformaldehyde at room temperature for 20 min. After washing in PBS, cells were blocked and permeabilized in PBS containing 2.5% goat serum and 0.1% Triton X-100 at room temperature for 90 min. Cells were incubated with human SHIP2 (1:25; Cell Technologies) overnight at 4°C. Detection was with Alexa Fluor 488 goat anti-rabbit IgG (1:500; Invitrogen) at room temperature for 1 h. As a negative control, antibodies against rabbit IgG were used. Cells were viewed and photographed with a Zeiss UV LSm 510 confocal microscope.

Normal human corneas were obtained from the Illinois Eye Bank. Frozen sections (5 μm) were fixed in 4% paraformaldehyde for 15 min at room temperature. After washing in PBS and blocking in PBS containing 2.5% BSA, sections were incubated overnight with SHIP2 (1:500) rabbit polyclonal antibody (ABGENT) at 4°C. As a negative control, sections were incubated with normal IgG. After washing, sections were sequentially incubated with biotinylated secondary anti-rabbit IgG, avidin-biotin-peroxidase (Vector), and diaminobenzidine tetrahydrochloride substrate (Sigma). Sections were counterstained with hematoxylin.

RNA Isolation and Northern Blots. We extracted total RNA from cells using TRIzol (Invitrogen). Total RNA was fractionated on a 15% denaturing (8 M urea) polyacrylamide gel, transferred to nylon membranes (Nytran N; Amersham Biosciences), and fixed by UV cross-linking. Membranes were probed with ^{32}P-labeled oligonucleotides complementary to miR-184 or miR-205. Hybridizations were carried out as described previously (1).

Western Blots. We extracted HeLa cells, HEKs, SCCs, and HCEKs with mammalian cell lysis Buffer (G-Biosciences) containing protease (G-Biosciences) and phosphatase (Calbiochem) inhibitors. Proteins from total cell lysates were resolved with a 4–20% Tris·HCl gradient gel (Bio-Rad), transferred to PVDF membranes, blocked in 5% nonfat milk in TBS/Tween 20, and blotted with antibodies for SHIP2 (Cell Signaling), phosphorylated Akt (Cell Signaling), Akt (Cell Signaling), phosphorylated BAD (Cell Signaling), BAD (Cell Signaling), phosphorylated PTEN (Cell Signaling), phosphorylated GSK-3β (Cell Signaling), and α-tubulin (Invitrogen).

1. Ryan DG, Oliveira-Fernandes M, Lavker RM (2006) MicroRNAs of the mammalian eye display distinct and overlapping tissue specificity. *Mol Vision* 12:1175–1184.

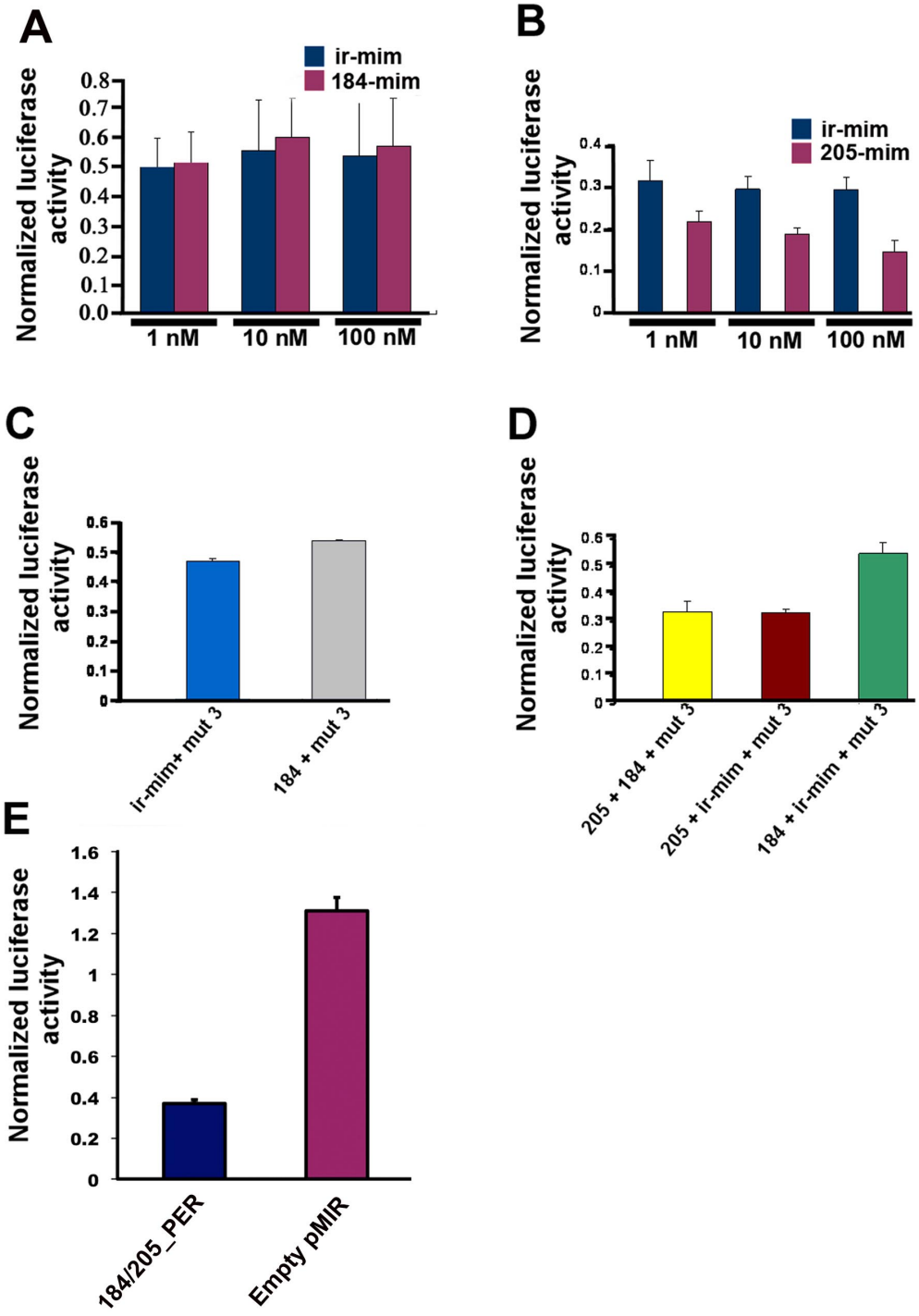

Fig. S1. Luciferase reporter assays showing effects of miR-184 and miR-205 on SHIP2 levels. (*A*) Luciferase activity of SHIP2 reporter in the presence of various concentrations (1–100 nM) of miR-184, showing that this miRNA cannot inhibit the luciferase activity of this construct. Error bars (SEM) are derived from three experiments in triplicate. (*B*) Luciferase activity of SHIP2 reporter in the presence of various concentrations (1–100 nM) of miR-205, showing the inhibitory activity of this reporter. Error bars (SEM) are derived from six experiments in triplicate. (*C*) Luciferase activity of mutated SHIP2 reporter (SHIP_mut3) cotransfected with either an irrelevant mimic (ir-mim + SHIP2_mut 3) or a miR-184 mimic (184 + SHIP2_mut 3), showing that cotransfections do not affect luciferase activity. Error bars (SEM) are derived from three experiments in triplicate. (*D*) Luciferase activity of mutated SHIP2 reporter (SHIP2_mut3) showing that cotransfection with either miR-184 mimic and miR-205 mimic (205 + 184 + SHIP2_mut3) or miR-205 mimic and an irrelevant mimic (205 + ir-mim + SHIP2_mut3) fail to restore luciferase activity of SHIP2 mutation 3. Conversely, cotransfection of miR-184 mimic and an irrelevant mimic (184 + ir-mim + SHIP_mut 3) does not affect luciferase activity. Error bars (SEM) are derived from three experiments in triplicate. (*E*) Luciferase activity of 184/205_PER and empty reporters in HEKs showing that endogenous miR-205 negatively regulates the positive control.

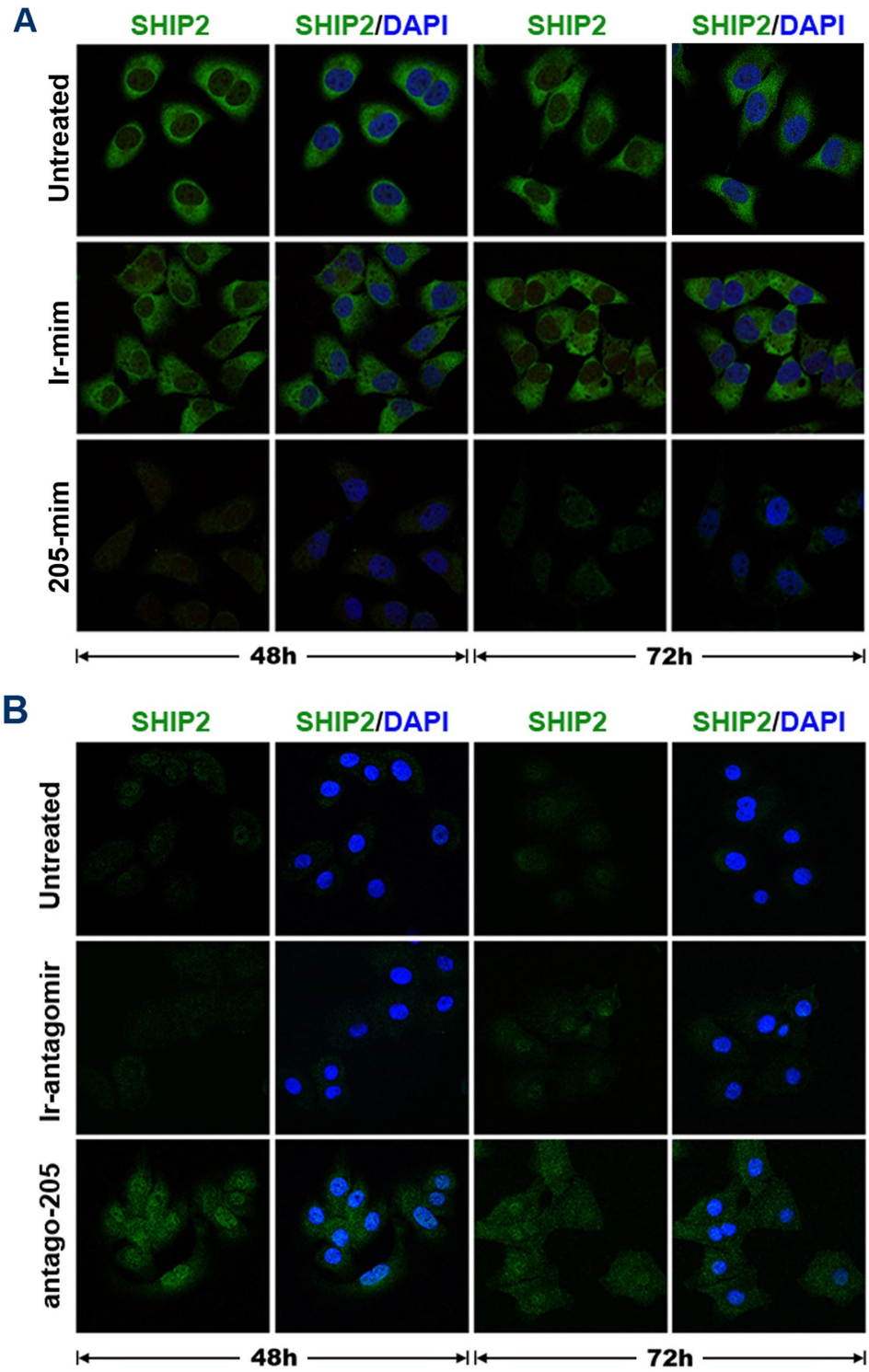

Fig. S2. (*A*) Immunofluorescence microscopy of HeLa cells stained with anti-SHIP2 and anti-SHIP2/DAPI showing a marked decrease in staining after 48 and 72 h of treatment with miR-205 mimic, compared with untreated cells and cells treated with an irrelevant mimic (ir-mim). (*B*) Immunofluorescence microscopy of HEKs stained with SHIP2 showing an increase in staining after 48 and 72 h of treatment with an antagomir to miR-205 (Antago-205), compared with untreated cells and cells treated with an irrelevant mimic (ir-antagomir).